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Stimulation and inhibition of adenylyl cyclases mediated by distinct regulatory proteins

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Adenylyl cyclases are under positive and negative control by guanine nucleotides and hormones[1-3]. Stimulatory responses are mediated by a guanine nucleotide- and Mg-binding regulatory component[4-6] (N_s), a protein that has been purified to homogeneity[7-9]. Inhibitory responses have been hypothesized[1-3,10,11] to be mediated by an analogous regulatory component (N_i) distinct from N_s, but definitive proof for this is lacking and these effects may result from modulation of N_s activity. Recently, *Bordetella pertussis* toxin has been shown[12] to ADP-ribosylate a peptide that is not part of N_s, and this coincides with attenuation of hormonal inhibition of adenylyl cyclase[12-17]. We show here that *cyc*⁻ S49 cells contain a substrate for ADP-ribosylation by pertussis toxin and that the toxin alters GTP dependent inhibition of *cyc*⁻ adenyl cyclase activity[18]. As *cyc*⁻ S49 cells do not contain N_s by several criteria[5,19-24], we conclude that N_i is a distinct and separate regulatory component of adenylyl cyclase.

Recently, we have shown that the *cyc*⁻ adenylyl cyclase is inhibited by GTP, other guanine nucleotides and NaF[18]. There was no information in this previous study, however, to suggest that these effects are mediated by the hypothetical N_i thought to confer hormonal inhibition to adenylyl cyclase in other systems[1-3]. These effects could, in fact, be due to a structurally altered N_s which functionally acts as an inhibitor of catalytic activity. To determine whether or not *cyc*⁻ contains an N_i, we have taken advantage of the recent observation that a toxin isolated from *B. pertussis* blocks hormone-mediated inhibition of adenylyl cyclase activity[12-17] and at the same time ADP-ribosylates a peptide of molecular weight (M_r) ~40,000 (40K) distinct from the N_s subunit which is a substrate for cholera toxin[12] (There are actually several toxins produced by *B. pertussis*. The one used in these studies, and referred to as pertussis toxin, is that characterized by Ui and collaborators[12-14] and referred to by them as islet activating protein.) At present, the function of the 40K peptide is unclear, but it does not seem to be a component of purified N_s (refs 7–9). It is either a component of N_i, if this does in fact exist, and interacts directly with the catalytic component of the system, or it is a regulatory element that modulates the activity of N_s and/or inhibitory receptors without interacting directly with the catalytic component of the enzyme. In the experiments presented here, we reasoned that if N_i is distinct from N_s, and if its presence in *cyc*⁻ membranes is the reason for guanine nucleotide regulation of *cyc*⁻ adenylyl cyclase activity[18], these membranes should contain a pertussis toxin substrate. More importantly, however, treatment of *cyc*⁻ cells with pertussis toxin should alter the pattern of inhibitory responses previously described[18]. On the other hand, if *cyc*⁻ membranes contain an altered N_s, these cells may or may not contain a pertussis toxin substrate, but treatment of *cyc*⁻ cells with toxin would not affect the response of *cyc*⁻ adenylyl cyclase to guanine nucleotides.

Figure 1 shows an autoradiogram from an experiment in which *cyc*⁻ S49 cell membranes were incubated with ^{32}P-NAD⁺ and various combinations of cholera toxin, pertussis toxin and purified N_s from human erythrocytes (J.C., J.D.H., R.I., J. Bryan and L.B., in preparation). With ^{32}P-NAD⁺ alone only a few high molecular weight proteins were labelled (Fig. 1, lane 1). In the presence of cholera toxin, there was nonspecific labelling of all the major protein bands (Fig. 1, lane 2), but no specific labelling of a 42–45K peptide characteristic of almost all intact adenylyl cyclase systems, including adipocytes[25], wild-type S49 cells[22], human[26], frog[27], pigeon[28,29], rat[30] and turkey[31] erythrocytes, and liver cells[32]. This finding is in agreement with the lack of an effect of cholera toxin on inhibition of *cyc*⁻ S49 adenylyl cyclase by guanine nucleotides[18]. Although incubation of *cyc*⁻ cell membranes with purified N_s alone (Fig. 1, lane 6) did not substantially alter the incorporation of ^{32}P-NAD⁺, the presence of purified N_s and cholera toxin together (lane 5) resulted in the specific labelling of a 42K peptide. Incubation of *cyc*⁻ S49 cell membranes with pertussis toxin and ^{32}P-NAD⁺

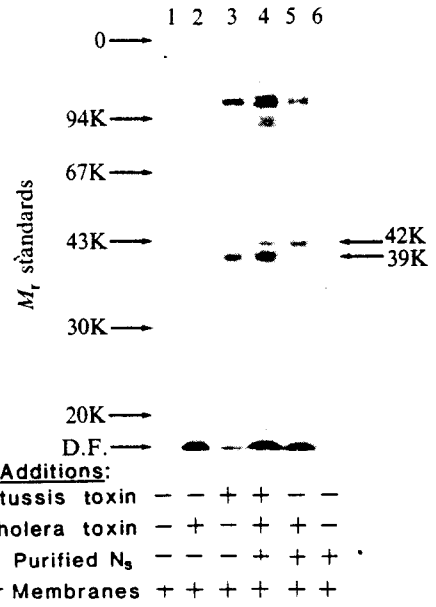

Fig. 1 Incorporation of [^{32}P]ADP-ribose into *cyc*⁻ S49 cell membrane proteins and purified human erythrocyte N_s. The additions to each sample are indicated. Lane 4 contains a mixture of the samples in lanes 3 and 5 and is included to show that the pertussis toxin substrate in *cyc*⁻ cell membranes is different from the cholera toxin substrate that is part of the purified N_s. *cyc*⁻ S49 cells from the M3B1 strain were grown according to the methods of Bourne *et al.*[36] and purified membranes prepared by the procedure of Ross *et al.*[24], except that Mg-free buffers were used throughout. Membranes (200 μg) were incubated in a final volume of 100 μl containing 1 mM ATP, 0.5 mM GTP, 15 mM thymidine, 100 mM potassium phosphate *p*H 7.5, 5 mM dithiothreitol (DTT) and 20 μM [^{32}P]NAD⁺ (specific activity 500 mCi mmol^{-1}). Incubations were for 30 min at 32 °C. Samples were then diluted fivefold with 10 mM Tris-HCl *p*H 7.5, 1 mM EDTA and the membranes precipitated by centrifugation at 23,000 r.p.m. for 60 min. The supernatant was removed and the membranes were resuspended in Laemmli's sample buffer[37] with 5% β-mercaptoethanol and allowed to stand at room temperature overnight. Samples were electrophoresed in 10% SDS-polyacrylamide gels made by the procedures of Laemmli[37]. An autoradiogram of the dried gel is shown. Concentrations, when present, were cholera toxin (Sigma), 20 μg ml^{-1}; pertussis toxin, 10 μg ml^{-1}; and purified erythrocyte N_s, 0.5 μg ml^{-1}. The N_s preparation was about 95% pure and consisted of two peptides of M_r 42,000 and 35,000. It was stored in 0.1% lubrol, 20 mM β-mercaptoethanol, 50 mM NaCl, 10 mM Na-HEPES *p*H 7.5, 30% ethylene glycol at −70 °C and diluted 30-fold for the assay. *B. pertussis* toxin was prepared from cultures of strain 114 (ref. 39) by a modification (R.D.S. and C.R.M., in preparation) of published procedures[39,40]. Pertussis toxin was preactivated by incubating it in 20 mM DTT for 30 min at 32 °C. Preactivation of cholera toxin is described elsewhere[18].

Table 1 Effect of 8 h pertussis toxin treatment of *cyc*⁻ S49 cells on adenylyl cyclase activity and inhibitory responses of cell menbranes

	Adenylyl cyclase activity (pmol mg^{-1} min^{-1})		% Inhibition		
	$MnCl_2$	$MgCl_2$ + forskolin	GTP	NaF	GMP-P(NH)P
Control	5.3 ± 1.6*	54.0 ± 16.1	17.9 ± 1.7	12.9 ± 4.8	42.7 ± 1.7
Toxin-treated	6.4 ± 2.1	79.0 ± 29.2	−4.3 ± 2.2	19.4 ± 4.4	38.3 ± 1.2
$\bar{d}$ ± s.e.m.	−1.1 ± 0.6	−25.0 ± 13.5	22.1 ± 1.1	−6.6 ± 3.3	4.4 ± 2.4
t(d.f. = 6)	−1.8	−1.9	20.1	2.0	1.8
P	NS	NS	< 0.001	NS	NS

The NaF concentration in the assays was 10 mM; the other conditions of the treatments and assays are described in the text and in Fig. 2 legend. Tests of statistical significance were done using a paired t-test. $\bar{d}$, Mean difference between control and toxin-treated values; NS, not significant.

* Values are mean ± s.e.m. from seven replicate experiments.

resulted in the labelling of a ~39K peptide (lane 3) which was a separate and distinct protein from the N_s subunit labelled by cholera toxin (lane 4).

The above experiment demonstrates that *cyc*⁻ S49 cells contain a pertussis toxin substrate, and that in this respect they resemble other cells possessing an inhibitory regulatory system that affects adenylyl cyclase[12–17]. To determine whether this substrate is functionally related to inhibition of adenylyl cyclase,

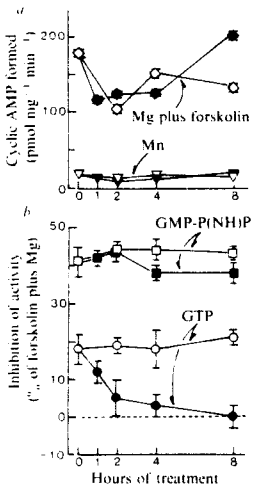

Fig. 2 Effect of treatment of *cyc*⁻ S49 cells with pertussis toxin on their adenylyl cyclase activity. *cyc*⁻ S49 cells were resuspended in fresh culture medium[36] at a density of 9.2×10^6 cells ml^{-1} without (control; open symbols) or with 0.1 μg ml^{-1} pertussis toxin (solid symbols). By 8 h of incubation control cultures had increased to 10.7×10^6 cells ml^{-1} and toxin-treated cultures to 9.7×10^6 cells ml^{-1}. At the indicated times membranes were prepared as described in Fig. 1 legend, except that the procedure was stopped after obtaining the 43,000g pellet. Adenylyl cyclase assays were done in a final volume of 50 μl containing ~10 μg membrane protein, 0.1 mM ATP (5×10^6 c.p.m. [α-^{32}P]ATP), 10 mM $MnCl_2$ or $MgCl_2$, 25 mM Na-HEPES *p*H 8.0, 1 mM EDTA, 1 mM cyclic AMP (10,000 c.p.m. [^{3}H⁻]cAMP) and a nucleoside triphosphate regenerating system composed of 20 mM creatine phosphate, 0.2 mg ml^{-1} creatine phosphokinase and 0.02 mg ml^{-1} myokinase. When present, forskolin (Calbiochem) was 100 μM, GTP 10 μM, and GMP-P(NH)P 10 μM. All other procedures were as described recently[18]. In *a* values are mean ± s.d. of three determinations. In *b*, per cent inhibition was calculated as $100(1 - F/T)$ where F is the mean of triplicate determinations in the presence of forskolin alone and T is that for forskolin plus GTP or GMP-P(NH)P. The error bars in *b* represent s.d.s derived from the following equation[41]:

$$\text{s.d.} = \left(\frac{T}{C}\right)\sqrt{\left(\frac{\text{s.d.}_C}{C}\right)^2 + \left(\frac{\text{s.d.}_T}{T}\right)^2}$$

cyc⁻ S49 cells were incubated in the presence of the toxin for 1–8 h (Fig. 2). In parallel, control cells incubated without toxin were collected at 0, 2, 4 and 8 h after starting the experiment. At each time point, membranes were prepared for adenylyl cyclase assays as described previously[18]. Adenylyl cyclase activities in the presence of either Mn or Mg plus forskolin were measured, as well as the ability of GTP and GMP-P(NH)P to inhibit the Mg plus forskolin-stimulated activity. Forskolin was included in the assays in the presence of Mg because it dramatically increases the measurable activity observed, although it is not required for obtaining the inhibitory effects of guanine nucleotides or NaF[18]. Figure 2*b* shows that, although the degree of inhibition by GMP-P(NH)P was affected little by toxin treatment, GTP inhibition of *cyc*⁻ S49 cell membrane adenylyl cyclase decreased with time of treatment. By 8 h of incubation with the toxin there was essentially no inhibitory effect of GTP.

Table 1 summarizes the results of seven experiments in which *cyc*⁻ S49 cells were incubated for 8 h in the absence (control) or presence of 0.1 μg ml^{-1} pertussis toxin. The actual magnitude of the specific activity of adenylyl cyclase in these separate experiments varied by as much as 10-fold, regardless of whether activities were determined in the presence of $MnCl_2$ or $MgCl_2$ plus forskolin. This variability, which is associated with the individual growth of *cyc*⁻ cells, seems to be related to the batch of horse serum available and the growth state of the cells at the moment of collection. Specific adenylyl cyclase activities of separate membrane batches derived from a single growth of cells, such as shown in Fig. 2, varied considerably less (never by more than twofold) and did not correlate with any known variable. Despite this variation in specific activity of the adenylyl cyclase in the membranes we prepared, the degree of inhibition by GTP, NaF or GMP-P(NH)P in membranes from control cells was relatively constant (Table 1). Moreover, as in Fig. 2, the results summarized in Table 1 show a consistent effect of pertussis toxin on GTP-mediated inhibition, reducing it to a level not significantly different from 0, with no significant change in the effects of NaF or GMP-P(NH)P.

To determine whether there is a causal relationship between the ADP-ribosylation of the 39K peptide in *cyc*⁻ membranes by pertussis toxin and the effect of the toxin on intact cells, the following experiment was performed. *cyc*⁻ S49 cell membranes from cells treated for 8 h with pertussis toxin, or control cells incubated without toxin, were incubated with ^{32}P-NAD⁻ in the presence or absence of pertussis toxin. Figure 3 shows that in control cell membranes there was, once again, a 39K peptide labelled by the toxin (compare lane 2 with lane 1). Membranes from cells previously exposed to pertussis toxin, however, no longer had an ADP-ribosylatable band at M_r 39,000, suggesting that at least one of the effects of the toxin on intact *cyc*⁻ S49 cells was to ADP-ribosylate this peptide. In this experiment, which used a higher pertussis toxin concentration and a lower membrane concentration than that in Fig. 1, significant labelling of ~29K peptide, as well as minor labelling of a ~26K peptide,

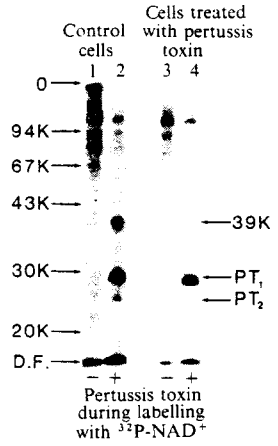

Fig. 3 Effect of pretreatment with pertussis toxin on subsequent labelling with ^{32}P-NAD$^+$ of *cyc*$^-$ S49 cells in the presence of toxin. *cyc*$^-$ S49 cells were incubated without (control) or with pertussis toxin for 8 h (as described in Fig. 2 legend) and membranes prepared as described in Fig. 1 legend. The conditions for pertussis toxin treatment of the membranes were the same as in Fig. 1 except that ~20 μg of membrane protein and 25 μg ml^{-1} toxin were used in the incubation.

was seen in both toxin-treated and control membranes subsequently incubated with pertussis toxin. These are two of the pertussis toxin subunits (PT_1 and PT_2 in Fig. 3) which are autolabelled in the conditions of the experiment (R.D.S. and C.R.M., unpublished observation).

Thus, the data presented here substantiate the hypothesis that N_i is a regulatory component of adenylyl cyclase which is separate and distinct from N_s. By at least five criteria, *cyc*$^-$ cells do not contain N_s. Their adenylyl cyclase is stimulated neither by guanine nucleotides nor by NaF[5,19], nor is it affected by cholera toxin treatment[18,20]; they contain neither radioimmunoassayable 42–45K subunits of N_s (ref. 21) nor cholera toxin substrate for ADP-ribosylation[22] and they lack β-adrenergic receptor regulation by guanine nucleotides and Mg (refs 23, 24). The only data which suggest that *cyc*$^-$ cells might contain an altered form of N_s are the findings that guanine nucleotides and NaF inhibit *cyc*$^-$ cell membrane adenylyl cyclase activity[18]. However, the results of this study correlate the inhibitory effects in the *cyc*$^-$ system with those seen in other biologically relevant systems where hormones inhibit adenylyl cyclase[12–17]. In both cases, pertussis toxin treatment leads to attenuation of inhibition and the concurrent ADP-ribosylation of a ~40K peptide. The presence of pertussis toxin substrates in other intact systems (that is, those having a functional N_s) suggests the existence of a N_i (ref. 12) (as do the kinetic arguments for the separate regulation of inhibitory and stimulatory responses[1,10,11]), but does not constitute proof of this hypothesis as these substrates may represent previously unrecognized subunits of N_s or receptor-related proteins which modulate the activity of N_s. As *cyc*$^-$ S49 cells do not contain N_s (refs 5, 19–24), the N_i-like activity seen here must be regulating the adenylyl cyclase catalytic unit directly and therefore must represent a transmembrane signalling protein different from N_s. This does not imply that N_i and N_s do not influence each other's ability to affect adenylyl cyclase, or that they may not compete for a similar binding site on the catalytic unit, but only that the two types of regulation of adenylyl cyclase reside in the properties of separate regulatory proteins.

The inhibitory effects seen in *cyc*$^-$ indicate several very striking similarities between N_i and N_s: both proteins are regulated by NaF as well as by guanine nucleotides[18], and in both cases the nonhydrolysable guanine nucleotide analogoues are more active than GTP, the natural effector. For both, hormonal effects correlate with stimulation of a low K_m GTPase activity[33–35]. In both cases the regulatory proteins are affected by an ADP-ribosylating toxin which causes an increase in the cyclic AMP content of their respective target cells. At the molecular level, the effects of these toxins alter GTP-mediated but not GMP-P(NH)P-mediated regulation. In one important respect, however, the effects of pertussis toxin on N_i differ markedly from those of cholera toxin on N_s. Whereas cholera toxin enhances GTP effects so that they are equivalent to those of GMP-P(NH)P, pertussis toxin reverses the effects of GTP on N_i without altering GMP-P(NH)P effects. Thus there are subtle but important differences in the mechanisms of action of N_i and N_s in their regulation of adenylyl cyclase and their mediation of hormonal effects.

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